Phytochemistry, Vol. 26, No. 4, pp. 933–937, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

PARTIAL PURIFICATION AND CHARACTERIZATION OF A LUTEOLIN-TRIGLUCURONIDE-SPECIFIC β-GLUCURONIDASE FROM RYE PRIMARY LEAVES (*SECALE CEREALE*)

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(*Received* 16 *June* 1986)

Key Word Index—*Secale cereale*; Poaceae; rye; luteolin triglucuronide turnover; β-glucuronidase.

Abstract—Primary leaves of rye contain a β-glucuronidase with high specificity for luteolin 7-*O*-[β-D-glucuronosyl (1 → 2)β-D-glucuronide]-4′-*O*-β-D-glucuronide, the major flavonoid of the leaf. The enzyme hydrolyses the glucuronic acid moiety in position 4′ (K_m = 7 μM; V_{max} = 1093 μkat/kg protein). A 330-fold purification was obtained by protein fractionation with ammonium sulphate, Sephadex G 150, hydroxylapatite and CM-Sepharose CL-6B. The enzyme has a pH optimum of 4.3 in 0.01 M citrate buffer. The molecular weight was determined to be 280 kD with active subunits of 67 kD. Isoelectric focusing indicates subunits of different isoelectric points at pH 5.5 and 6.3. The β-glucuronidase shows a temperature optimum at 55° and is inhibited by heavy metal ions such as Cu (0.85 mM) and Ag (0.25 mM), but is activated by ethyleneglycol monomethylether up to 15%. The enzyme is stable in 50% glycerol at −20° for at least 2 months. The results suggest that this β-glucuronidase is involved in the turnover of luteolin triglucuronide *in vivo*.

INTRODUCTION

In some plants, flavonoid glycosides undergo endogenous catabolism or turnover which is initiated by deglycosylation [1–6]. While β-glucosidases are known with a high specificity for phloridzin and isoflavone 7-*O*-glucosides, enzymes with a similar substrate specificity have not been described for other flavonoid glycosides [7]. In previous papers we reported on the tissue localization of two major luteolin *O*-glucuronides in the mesophyll of rye primary leaves, *Secale cereale* L. [8, 9]. These flavonoids exhibit characteristic dynamics of biosynthesis, accumulation and turnover during leaf development. Luteolin 7-*O*-[β-D-glucuronosyl(1 → 2)β-D-glucuronide]-4′-*O*-β-D-glucuronide decreases drastically after the fifth day of development. This loss is obviously due to turnover via luteolin 7-*O*-diglucuronide. In the present paper we show that a β-glucuronidase catalyses the removal of glucuronic acid in the 4′-position as the first step.

β-Glucuronidases from animals and micro-organisms are known and their enzymology is well investigated. Only a few studies, mostly using crude enzyme extracts, have been carried out with β-glucuronidases from plants [10–15]. In this report we present for the first time the partial purification and characterization of a highly specific plant β-glucuronidase from rye primary leaves.

RESULTS

Six- or seven-day-old rye primary leaves are suitable for β-glucuronidase extraction and purification because the enzyme has reached its maximal activity at this stage of leaf development (data not shown). In crude extracts, β-glucuronidase shows optimal stability in 0.05–0.1 M neutral or weakly acidic buffers such as phosphate and citrate, pH 5–7. Therefore, these conditions were used for extraction and for time-consuming procedures such as chromatography on Sephadex G 150. Under alkaline conditions the enzyme suffered irreversible loss of activity. Alkaline lability has been reported for some glycosidases, e.g. for β-glucosidases from *Aspergillus aculeatus* [16]. A suitable procedure for purification of rye β-glucuronidase was gel filtration on Sephadex G 150, followed by chromatography on hydroxylapatite and then on CM-Sepharose CL-6B. Furthermore, the slowly performed pH decrease (from pH 7.0 to 6.0 for chromatography on hydroxylapatite and from pH 6.0 to 5.0 on CM-Sepharose CL-6B) led to precipitation of accompanying proteins. On Sephadex G 150, the β-glucuronidase activity was highest at the elution volume near 210 ml, where the bulk of protein was already eluted (Fig. 1). After concentration of the active fractions, 76% of the original enzyme activity was retained. Further purification was achieved by chromatography on hydroxylapatite, where the β-glucuronidase activity eluted in a symmetrical peak (Fig. 2). Purification of this peak by CM-Sepharose CL-6B (Fig. 3) resulted in a final enrichment of *ca* 330-fold. The losses of activity during the two last chromatographic steps are due to instability of the β-glucuronidase in solutions of low protein concentration. The purification procedure is detailed in Table 1. The purified enzyme was stable without significant loss of activity for *ca* 2 months when stored at −20° in 50% glycerol.

Native and analytical SDS electrophoresis showed that the β-glucuronidase was not homogeneous. Native electrophoresis according to Maurer [17] showed in addition to the main protein band of β-glucuronidase several weaker bands without β-glucuronidase activity. In the case of SDS electrophoresis, a very weak band above

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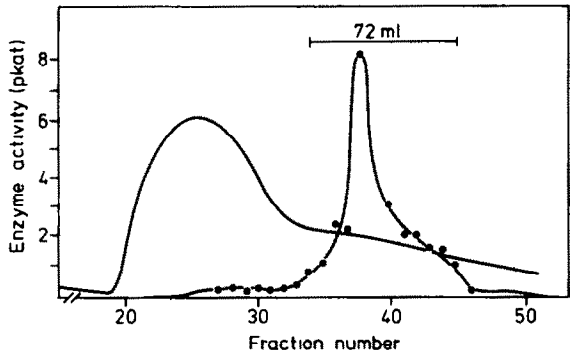

Fig. 1. Elution profile of β-glucuronidase activities (●) from Sephadex G 150; elution buffer 0.05 M K-Pi, pH 7.0. (——) $A_{280\,nm}$. The bar indicates the fractions pooled for further purification.

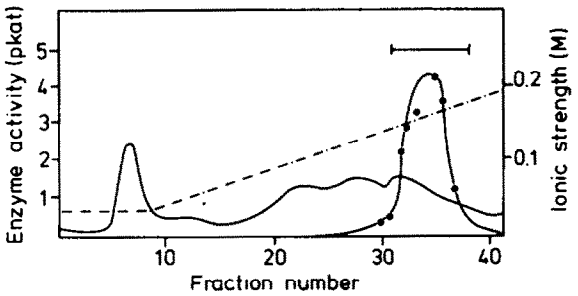

Fig. 2. Elution profile of β-glucuronidase activities (●) from hydroxylapatite. (– · –) Elution with a linear gradient of K-Pi buffer, pH 6.0. (——) $A_{280\,nm}$. The bar indicates the fractions pooled for further purification.

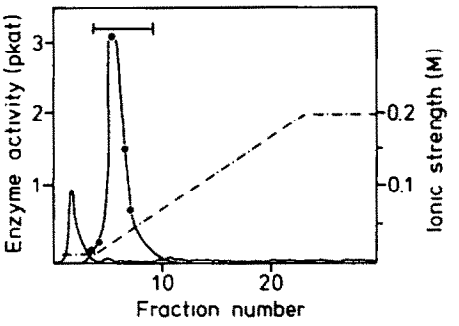

Fig. 3. Elution profile of β-glucuronidase activities (●) from CM-Sepharose CL-6B. (– · –) Elution with a linear gradient of citrate buffer, pH 5.0. (——) $A_{280\,nm}$. The bar indicates the fractions pooled, concentrated and stored for characterization.

the main band of β-glucuronidase was observed. Since Maurer's system is alkaline, which decreases enzyme activity, the acidic electrophoresis system of Davis [18] was also used. In Davis's system, only one protein band possessing β-glucuronidase activity was found. It is not clear whether the weaker bands of protein found in gels after alkaline electrophoresis are partly artefacts or if some accompanying proteins are not mobile when the acidic system is used.

Properties of the purified β-glucuronidase

pH optimum. The pH dependence was measured with citrate buffer of constant ionic strength. Like most plant glycosidases [19], rye β-glucuronidase possesses a pH optimum at 4.3, with half maximal activity at pH 2.1 and 5.8, respectively. A β-glucuronidase activity present in potato slices, involved in steroidal glycoside metabolism, showed the same apparent pH optimum [13], as does the β-glucuronidase found in the emulsin preparation from almonds [20]. However, neither of these latter enzymes has been purified.

Influence of ionic strength. The activity of the β-glucuronidase is dependent on the ionic strength. When incubated under standard conditions with buffers of various ionic strengths, the highest activity was found when buffers of low concentration were used. Maximal activity was observed with 10 mM buffer. Figure 4 shows the dependence of the activity for citrate buffer of different ionic strengths.

Time and temperature dependence. When purified β-glucuronidase was assayed at 30° the reaction was linear up to 40 min. This is in contrast to crude-extract measurements from rye primary leaves where linearity was observed for more than 6 hr. The β-glucuronidase showed a temperature optimum at 55°.

Figure 5 exhibits the corresponding Arrhenius plot; two slopes were observed with a break point at 35°. The energies of activation in the ranges 10–35° and 35–55° were 16.5 and 75.6 kJ/mol, respectively. Changes in the enzyme conformation could cause the biphasic behaviour. Temperatures above 55° led to rapid denaturation. Miwa [14] reports that a β-glucuronidase extracted from *Scutellaria baicalensis* showed various temperature coefficients depending on the temperature range, although he used an enzyme preparation of unknown degree of purification.

Substrate specificity and K_m *values.* Substrates similar to the physiological substrate, rye compound R_1, were tested in our β-glucuronidase assay. We did not use compounds which belonged to a different class of chemical structure such as nitrophenylglucuronide since the application of artificial substrates had often resulted in a low specificity towards the aglycone [21].

The results listed in Table 2 show a high specificity for the natural substrate, luteolin triglucuronide (R_1). Of the other flavonoid glycosides examined, only apigenin 7,4'-*O*-diglucuronide and luteolin 7-*O*-glucuronide were hydrolysed. In the case of apigenin 7,4'-*O*-diglucuronide, the glucuronic acid in the 4'-position was released; the product apigenin 7-*O*-glucuronide was hardly attacked at all (like the other flavone 7-*O*-glucuronides tested); the V_{max} of this reaction was 54% relative to the natural substrate R_1, while the K_m values were the same. In contrast to apigenin 7-*O*-glucuronide, luteolin 7-*O*-glucuronide was hydrolysed, but V_{max} was 64% and the K_m was three times higher compared to compound R_1. Luteolin 7-*O*-diglucuronide (compound R_2), the product of the deglucuronidation, which occurs naturally in rye primary leaves, was not attacked. The enzyme is apparently not able to cleave the bond between the two glucuronic acid moieties or hydrolyse them as an intact

Table 1. Purification of β-glucuronidase from rye primary leaves

Purification step	Activity (pkat)	Protein (mg)	Specific activity (μkat/kg)	Yield (%)	Purification (-fold)
Crude extract	11933	260	45.8	100	—
$(NH_4)_2SO_4$ (30–80% saturation) and Sephadex G 25	13733	210	65.4	115	1.4
First ultrafiltration	10550	191	55	88	1.2
Sephadex G 150 and ultrafiltration	9120	45	203	76	4.4
Hydroxylapatite, ultrafiltration and dialysis	3433	1.2	2861	29	62.5
CM-Sepharose-CL-6B and ultrafiltration	1516.7	0.1	15170	12	331

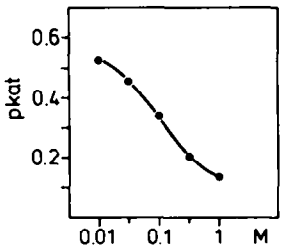

Fig. 4. Dependence of purified β-glucuronidase activity on the ionic strength. Citrate buffer, pH 4.3, 10–1000 mM.

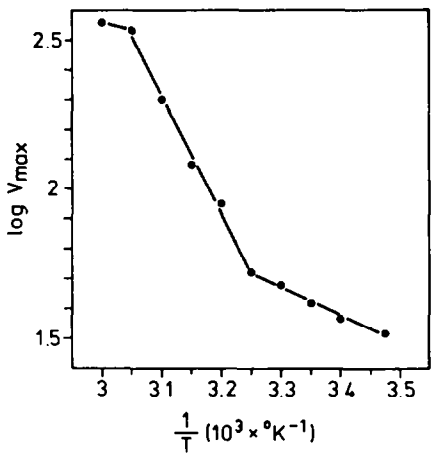

Fig. 5. Arrhenius plot for the determination of the apparent energy of activation of purified β-glucuronidase.

disaccharide unit. Furthermore, the β-glucuronidase was not capable of hydrolysing glycosides based on glucose, galactose or arabinose. In addition to the sugar moiety, the configuration of the aglycone seems principally to determine the substrate specificity. A β-glucosidase purified from *Cicer arietinum* showed comparable substrate specificity [6].

Inhibition and activation of β-glucuronidase. The enzyme was not inhibited by 10 mM concentrations of glucuronic acid lactone, glucose or UDP-glucuronic acid. D-Saccharic acid 1,4-lactone was an inhibitor, with 50% inhibition at 1 mM. Ca^{2+} and Mg^{2+} ions showed no effect, whereas Cu^{2+} and Ag^{+} ions inhibited to 50% at 0.85 and 0.25 mM, respectively. Up to 15% ethyleneglycol monomethylether, used as a solvent for lipophilic substrates, increased the activity by 30–50% compared to the control. Methanol showed an inhibiting effect of 10–40% in this range of concentration. Hösel and Nahrstedt [22] reported that β-glucosidases from *Alocasia macrorrhiza* are inhibited by Cu^{2+} and Ag^{+} ions but are activated by solvents with lipophilic properties such as ethyleneglycol monomethylether. 2-Mercaptoethanol or dithioerythritol had no influence on rye β-glucuronidase up to 10 mM.

Molecular weight and isoelectric point. Molecular weight determination of β-glucuronidase by Sephadex G 150 and SDS-gel electrophoresis showed an M_r of 67000 ± 10%. There are some indications that this molecular weight is that of two similar subunits. For example, analytical isoelectric focusing showed two bands of equal intensity with isoelectric points at pH 5.5 and 6.3. When crude extracts were concentrated by ultrafiltration instead of ammonium sulphate precipitation, and applied to the Sephadex G 150 column, two peaks of β-glucuronidase activity could be observed; the first one with an M_r of *ca* 280000 had 10–15% of the activity of the second peak having an M_r of 67000. The enzyme thus appears to be composed of two pairs of subunits, both with M_r 67000.

DISCUSSION

The properties of the purified β-glucuronidase from rye primary leaves are similar to those of most plant glycosidases described so far [19]. However, the enzyme shows a high specificity for the substrate luteolin 7-*O*-diglucuronide 4′-*O*-glucuronide as well as for the glucuronic acid moiety at the 4′-*O*-position. Rye β-glucuronidase exhibits a low K_m (high affinity) and a high V_{max} value for its natural substrate whereas the product of deglucuronidation, the naturally occurring luteolin 7-*O*-diglucuronide, is not attacked. This contrasts with the ability of the enzyme to hydrolyse luteolin 7-*O*-glucuronide with 64% efficiency. Concerning other flavonoid-specific β-glycosidases, comparable low K_m values are described only for a β-glucosidase specific for phloridzin from apple seeds, with a K_m of 14 μM [5], and for the isoflavone 7-*O*-glucoside-specific β-glucosidase of *Cicer arietinum* seedlings, where the K_m was in the range 20–30 μM [6].

EXPERIMENTAL

Plant material. Caryopses of *Secale cereale* L. were purchased from F. von Lochow-Petkus, Bergen (F.R.G.). The growing conditions of the seedlings have been described elsewhere [8].

Table 2. Substrate specificity of the β-glucuronidase

	Activity (%)	K_m (μM)	V_{max} (μkat/kg)
Luteolin 7-*O*-diglucuronide 4'-*O*-glucuronide (R_1)	100	7	1093.7
Luteolin 7-*O*-diglucuronide (R_2)	0	—	—
Luteolin 7-*O*-glucuronide	64	20	703
Apigenin 7,4'-*O*-diglucuronide	54	7	584
Apigenin 7-*O*-glucuronide	<1	—	—
Chrysoeriol 7-*O*-glucuronide	<1	—	—
Apigenin 7-*O*-apiosylglucoside (apiin)	0	—	—
Apigenin 7-*O*-glucoside	0	—	—
Isovitexin 2"-*O*-arabinoside	0	—	—
Isovitexin 2"-*O*-galactoside	0	—	—
Isovitexin 7-*O*-glucoside (saponarin)	0	—	—
Vitexin 2"-*O*-rhamnoside	0	—	—
Vitexin	0	—	—
Isoorientin 7-*O*-glucoside (lutonarin)	0	—	—
Quercetin 3-*O*-glucuronide	0	—	—

Other materials. Sephadex G 25, Sephadex G 150 and CM Sepharose CL-6B were obtained from Pharmacia. Hydroxylapatite Bio-Gel HTP and the SDS-page standard proteins were purchased from Bio-Rad. Standard proteins for analytical isoelectric focusing as well as for M_r determination by gel-sieve chromatography were obtained from Serva. All other chemicals were of analytical grade and used without further purification.

Preparation of substrates. Vitexin, apiin and apigenin 7-*O*-glucoside were obtained from Carl Roth and used as substrates after purification according to standard procedures [9]. Quercetin 3-*O*-glucuronide was a gift from Prof. E. Wollenweber, Darmstadt (F.R.G.). All other substrates were isolated from appropriate plant sources: saponarin and lutonarin from primary leaves of *Hordeum vulgare* [23]; apigenin 7-*O*-glucuronide, luteolin 7-*O*-glucuronide, apigenin 7,4'-*O*-diglucuronide and chrysoeriol 7-*O*-glucuronide from *Antirrhinum majus* petals [24]; vitexin 2"-*O*-rhamnoside and isovitexin 2"-*O*-arabinoside from primary leaves of *Avena sativa* [25]; luteolin 7-*O*-diglucuronide 4'-*O*-glucuronide (compound R_1) luteolin 7-*O*-diglucuronide (compound R_2) and isovitexin 2"-*O*-galactoside from primary leaves of *Secale cereale* [9, 26]. The general procedure of purification has been described elsewhere [8, 9].

Enzyme extraction and purification. All steps were carried out at 0–4°; buffers contained 1 mM 2-mercaptoethanol, unless otherwise stated. Chromatographic procedures were monitored for protein at 280 nm. 80–100 g of 6- to 7-day-old rye primary leaves were frozen in liquid N_2 and ground in a mortar. The powder was allowed to thaw in 600–800 ml 0.1 M phosphate buffer, pH 7.0, containing 50% (w/w) Dowex AG 1 × 2 (200–400 mesh, Cl^- form) and 15% (w/w) Polyclar AT. The mixture was stirred for 30–45 min, filtered through miracloth and centrifuged at 40000 *g* for 15 min. The supernatant containing the β-glucuronidase was taken as the crude extract and proteins were precipitated by adding solid $(NH_4)_2SO_4$. The fraction of 30–75% saturation was taken for further preparation. The pellet was redissolved in a minimal vol. of 0.1 M K-Pi buffer, pH 7.0, and desalted on a Sephadex G 25 column (2.5 × 30 cm). The protein fraction was concentrated to 10 ml by ultrafiltration using an Amicon (Danvers, MA, U.S.A.) ultrafiltration model 8050 cell and a 10 PM 30 ultrafiltration membrane. The concentrate was chromatographed on a Sephadex G 150 column (2.5 × 70 cm, equilibrated with 0.05 M K-Pi buffer, pH 7.0). Active fractions were combined and concentrated by ultrafiltration as described above. After dialysis against 0.01 M K-Pi buffer, pH 6.0, the extract was applied to a hydroxylapatite column (1.5 × 12 cm, equilibrated with 0.01 M K-Pi buffer, pH 6.0). The column was washed with equilibration buffer (35–40 ml) and the enzyme was eluted with a linear gradient of 10–200 mM K-Pi buffer, pH 6.0. Active fractions were combined and concentrated by ultrafiltration to a vol. of 2 ml. The enzyme was dialysed overnight against 0.01 M citrate buffer, pH 5.0, and then applied to a CM-Sepharose CL-6B column (1 × 10 cm, equilibrated with 0.01 M citrate buffer, pH 5.0). The column was washed twice with equilibration buffer and the β-glucuronidase was eluted with a linear gradient of 10–200 mM citrate buffer, pH 5.0. Active fractions were combined and concentrated to 2 ml by a final ultrafiltration step. The β-glucuronidase was stored at −20° in 50% glycerol. Protein was determined by Bradford's method [27], using BSA as standard.

Enzyme assay. The standard incubation mixture consisted of 30–300 μM flavonoid (dissolved in H_2O or in ethyleneglycol monomethylether), 0.01 M citrate buffer, pH 4.0, and 5–20 μl of enzyme in a total vol. of 100 μl. The reaction was started with substrate. After an incubation period of 30–60 min at 30°, the reaction was terminated by the addition of MeOH. Samples were stored at −20°. β-Glucuronidase activity was measured by HPLC.

HPLC system. 8800 Gradient Controller, chromatographic pump DuPONT Instruments, Bad Nauheim (F.R.G.). Absorbance detection: Spectroflow 773, detector Kratos Analytical Instruments, Trappenkamp (F.R.G.). Evaluation: SP 4270 Integrator, Spectraphysics, Santa Clara, CA (U.S.A.). Detection wavelength: 340 nm. The HPLC column was prepacked with Lichrosorb RP-8 from Bischoff, Leonberg (F.R.G.). Separation of product (compound R_2) and substrate (compound R_1) was accomplished within 10 min by elution with an isocratic system (77% H_2O containing 1% H_3PO_4, 12% MeOH, 4% MeCN and 7% THF). For separation of less polar substrates and

products two other isocratic systems were used: (1) 71% H_2O (1% H_3PO_4), 12% MeOH, 10% MeCN and 7% THF for di- and monoglycosides within 8 min; (2) for monoglycosides and aglycones: 67% H_2O (1% H_3PO_4), 11% MeOH, 12% MeCN, 10% THF, elution was for 15 min. For detection of unknown products, a 20 min linear gradient from 100% H_2O (1% H_3PO_4) to 100% MeCN was used.

Analytical polyacrylamide disc electrophoresis. Polyacrylamide disc electrophoresis was carried out according to Maurer [17] (system No. 1, Tris–glycine buffer, pH 8.1) or Davis [18] (Ala–HOAc buffer, pH 5.0). Gels were stained for protein with Coomassie brillant blue by the method of Reisner *et al.* [28]. β-Glucuronidase was detected by incubating the sliced gel in the standard assay.

SDS electrophoresis and M_r determination. SDS electrophoresis was performed as described by Laemmli [29] at 30 mA per gel at 4°, 3–4 hr, using a Protean TM II vertical electrophoresis system and a constant power supply model 3000/300 from Bio-Rad, Richmond, CA (U.S.A.). The low M_r protein standard, operating range 10000–100000 from Bio-Rad, was used for M_r estimation of subunits. Proteins were stained with Coomassie brillant blue as described above. The M_r of the β-glucuronidase was further estimated by gel sieve chromatography using Sephadex G 150. The column was calibrated with standard proteins in the range of 450–17 kD. Elution behaviour was determined by measuring absorption at 280 nm and β-glucuronidase activity.

Analytical isoelectric focusing. Gel isoelectric focusing was carried out according to O'Farrel [30] with Ampholine, pH 3.5–10, from LKB Instrument, Gräfelfing (F.R.G.). The pH gradient was determined by cutting the gels into 3 mm slices, treating the slices with 0.1 M KCl solution for 3 hr, and the pH of each slice was measured. A set of reference proteins from Serva was used as markers. Isoelectric points were estimated from the pH gradient by the position of protein bands.

Acknowledgements—Financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged. We are grateful to Profs. G. Legler (Köln) and J. W. McClure (Oxford, OH, U.S.A.) for valuable suggestions and for revising the English version of the manuscript.

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